

Kondrashova, O. et al. (2017) Secondary somatic mutations restoring RAD51C and RAD51D associated with acquired resistance to the PARP inhibitor rucaparib in high-grade ovarian carcinoma. *Cancer Discovery*, (doi:[10.1158/2159-8290.CD-17-0419](https://doi.org/10.1158/2159-8290.CD-17-0419))

This is the author's final accepted version.

There may be differences between this version and the published version. You are advised to consult the publisher's version if you wish to cite from it.

<http://eprints.gla.ac.uk/141955/>

Deposited on: 05 June 2017

Secondary somatic mutations restoring *RAD51C* and *RAD51D* associated with acquired resistance to the PARP inhibitor rucaparib in high-grade ovarian carcinoma

Olga Kondrashova^{1,2*}, Minh Nguyen^{3*}, Kristy Shield-Artin^{1,2*}, Anna V. Tinker⁴, Nelson N.H. Teng⁵, Maria I. Harrell⁶, Michael J. Kuiper⁷, Gwo-Yaw Ho^{1,2,8}, Holly Barker¹, Maria Jasin⁹, Rohit Prakash⁹, Elizabeth M. Kass⁹, Meghan R. Sullivan¹⁰, Gregory J. Brunette¹⁰, Kara A. Bernstein¹⁰, Robert L. Coleman¹¹, Anne Floquet¹², Michael Friedlander¹³, Ganessan Kichenadasse¹⁴, David M. O'Malley¹⁵, Amit Oza¹⁶, James Sun¹⁷, Liliane Robillard³, Lara Maloney³, David Bowtell on behalf of the AOCS Study Group¹⁸⁻²⁰, Heidi Giordano³, Matthew J. Wakefield^{1,7}, Scott H. Kaufmann²¹, Andrew D. Simmons³, Thomas C. Harding³, Mitch Raponi³, Iain A. McNeish²², Elizabeth M. Swisher⁶, Kevin K. Lin^{3*}, Clare L. Scott^{1,2,8*,†}

Affiliations:

¹Walter and Eliza Hall Institute of Medical Research, Melbourne, VIC, Australia

²Department of Medical Biology, The University of Melbourne, Melbourne, VIC, Australia

³Clovis Oncology, Inc., Boulder, CO, United States of America

⁴British Columbia Cancer Agency, Vancouver, BC, Canada

⁵Stanford University, Palo Alto, CA, United States of America

⁶University of Washington, Seattle, WA, United States of America

⁷Melbourne Bioinformatics, The University of Melbourne, Melbourne, VIC, Australia

⁸Department of Medical Oncology, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia.

⁹Developmental Biology Program, Memorial Sloan Kettering Cancer Center, New York, NY, United States of America

¹⁰Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, United States of America

¹¹The University of Texas MD Anderson Cancer Center, Houston, TX, United States of America

¹²Institut Bergonié, Bordeaux, France

¹³University of New South Wales and Prince of Wales Hospital, Sydney, NSW, Australia

¹⁴Flinders University, Adelaide, SA, Australia

¹⁵The Ohio State University, James Cancer Center, Columbus, OH, United States of America

¹⁶Princess Margaret Cancer Centre, University Health Network, Toronto, ON, Canada

¹⁷Foundation Medicine, Inc., Cambridge, MA, United States of America

¹⁸Peter MacCallum Cancer Centre, Melbourne, VIC, Australia

¹⁹Centre for Cancer Research, University of Sydney at Westmead Millennium Institute, and Departments of Gynaecological Oncology, Westmead Hospital, Sydney, NSW, Australia

²⁰QIMR Berghofer Medical Research Institute, Brisbane, QLD, Australia

²¹Mayo Clinic Cancer Centre, Rochester, MN, United States of America

²²Institute of Cancer Sciences, University of Glasgow, Glasgow, United Kingdom

* Contributed equally to this work

†Corresponding author: scotte@wehi.edu.au (CLS); Walter and Eliza Hall Institute of Medical Research, 1G Royal Parade, Parkville, Victoria, 3052, Australia. (tel) +61 3 9345 2498 (fax) +61 3 9347 0852

Running title: Secondary mutations in *RAD51C* and *RAD51D*

Keywords: Serous ovarian cancer, PARP inhibitor, reversion mutation, DNA repair, BRCA1, RAD51C, RAD51D

Conflict of Interest:

CLS's institution received in kind research support for parallel laboratory work using rucaparib.

M. Nguyen, L. Robillard, L. Maloney, H. Giordano, A.D. Simmons, T.C. Harding, and K. Lin are employees of Clovis Oncology, Inc., and hold stock in Clovis Oncology, Inc. M. Raponi holds stock in Clovis Oncology, Inc. J. Sun is an employee of Foundation Medicine, Inc.

Abstract

High-grade epithelial ovarian carcinomas (OC) containing mutated *BRCA1* or *BRCA2* (*BRCA1/2*) homologous recombination (HR) genes are sensitive to platinum-based chemotherapy and poly(ADP-ribose) polymerase inhibitors (PARPi), while restoration of HR function due to secondary mutations in *BRCA1/2* has been recognized as an important resistance mechanism. We sequenced core HR pathway genes in 12 pairs of pre-treatment and post-progression tumor biopsy samples collected from patients in ARIEL2 Part 1, a phase 2 study of the PARPi rucaparib as treatment for platinum-sensitive, relapsed OC. In six of 12 pre-treatment biopsies, a truncation mutation in *BRCA1*, *RAD51C* or *RAD51D* was identified. In five of six paired post-progression biopsies, one or more secondary mutations restored the open reading frame. Four distinct secondary mutations and spatial heterogeneity were observed for *RAD51C*. *In vitro* complementation assays and a patient-derived xenograft (PDX), as well as predictive molecular modeling, confirmed that resistance to rucaparib was associated with secondary mutations.

Statement of Significance

Analyses of primary and secondary mutations in *RAD51C* and *RAD51D* provide evidence for these primary mutations in conferring PARPi sensitivity and secondary mutations as a mechanism of acquired PARPi resistance. PARPi resistance due to secondary mutations underpins the need for early delivery of PARPi therapy and for combination strategies.

Introduction

The hallmark of synthetic lethality is the requirement for two complementary hits that, while tolerated individually, result in cancer cell death when they occur together. A prime example is the observation that cells tolerate poly(ADP-ribose) polymerase (PARP) inhibition or homologous recombination (HR) impairment individually, but cancer cells with impaired HR are killed by PARP inhibitors (PARPi), reflecting drug-induced inhibition of PARP1 catalytic activity, trapping of PARP1 at sites of DNA damage, and/or alterations in the balance between error-free and error-prone repair pathways (1-5).

An exquisite proof of synthetic lethality comes from high-grade epithelial ovarian carcinomas (OC) with mutated *BRCA1* or *BRCA2* (*BRCA1/2*) that are sensitive to platinum-based chemotherapy and PARPi (6-9). Furthermore, somatic reversion mutations in either *BRCA1/2* following exposure of OC to platinum-based chemotherapy or PARPi are identified as a mechanism of resistance. First reported in 2008 in a human pancreatic cell line and human OC (10,11), secondary mutations that restore the wild type *BRCA2* open reading frame were detected in clinical OC, with a higher rate in women with platinum-resistant OC who had prior chemotherapy (12,13). More recently, examination of multiple tumor deposits at autopsy revealed additional evidence of *BRCA2* reversion mutations and intra-patient heterogeneity with 12 distinct reversion events observed in a single patient with end-stage *BRCA2*-mutant OC who had received multiple chemotherapy regimens (14). To date, most of the secondary *BRCA1/2* mutations are documented after platinum-chemotherapy exposure, with only limited reports after PARPi (15).

Identification of patients with wild-type *BRCA1/2* but HR-defective OC is important as these patients may potentially respond to PARPi therapy. Germline or somatic mutations in core HR genes beyond *BRCA1/2*, although individually rare, collectively occur in 7-8% of OC (16,17) and have been shown *in vitro* and in patients to underpin responses to PARPi (9,18). These mutations

are being increasingly identified in the clinic due to use of germline multiplex genetic testing and tumor sequencing (16,19). In addition to *BRCA1/2*, the RAD51 paralogues *RAD51C* and *RAD51D* are well established core HR pathway genes in which germline mutations increase ovarian cancer susceptibility (20-24). We recently reported that the PARPi rucaparib is active in OC with *RAD51C* or *RAD51D* mutations, with 3 partial responses (PR) and two cases of prolonged stable disease (SD) for 8.3 and 11.0 months duration among five evaluable patients treated with rucaparib (9). It is unknown if somatic reversion mutations are a mechanism of acquired resistance in cancers driven by mutations in HR genes beyond *BRCA1/2*.

Here, we investigated whether secondary mutations in genes other than *BRCA1/2* can arise as a mechanism of resistance post exposure to the PARPi rucaparib.

Results

To study acquired PARPi resistance, we profiled pre-treatment tumor samples and post-progression biopsies from twelve patients with platinum-sensitive, relapsed high-grade epithelial OC treated with rucaparib in ARIEL2 Part 1. Samples were assessed using targeted next generation sequencing (NGS) with Foundation Medicine's T5 assay, which sequences 287 cancer-related genes, including core HR pathway genes (Supplementary Fig. 1, Supplementary Table 1; ref. 9). In six of the 12 cases, a deleterious mutation causing early protein termination in an HR pathway gene (four in *BRCA1* and one each in *RAD51C* and *RAD51D*; five germline and one somatic mutation) was detected in either an archival tumor sample (n=6) and/or screening biopsy sample (n=4) prior to initiation of rucaparib treatment (Table 1). All six patients with HR pathway genes mutated in their OC derived clinical benefit from rucaparib (four with a confirmed RECIST PR and two with stable disease; PFS ranged from 9.6 to 22.0 months). In a seventh case, a somatic *CDK12* mutation (c.264delC) was identified with no additional mutations detected in the post-progression biopsy. *CDK12* has been reported to impact transcription of multiple HR genes, although is yet to be established as a core HR pathway gene (25, 26).

In five of six cases with HR pathway gene mutations, post-progression biopsy samples contained at least one secondary mutation that was not detected in the pre-treatment carcinomas. These secondary mutations restored the open reading frame of the HR genes and thus potentially restored HR function and conferred resistance to rucaparib (Table 1). In case 2, the only case in which no secondary HR gene mutation was detected in the post-progression biopsy sample, the possibility of a reversion mutation to wild-type sequence was unlikely, because the wild-type allele frequency observed was not higher than expected based on the estimated tumor purity. The secondary mutations detected in the *BRCA1*-mutated cases were large in-frame deletions (ranging from 123 to 861bp) that restored the open reading frame either by deleting the primary frameshift mutation (cases 1 and 3), or by shifting the reading frame back into the correct state (case 4). The phasing of

primary and secondary mutations in case 4 could not be established with the Foundation Medicine's T5 assay, since the secondary mutation (c.1835_1964del) was 80bp away from the primary mutation (c.2043dup); therefore, we performed colony PCR that confirmed cis configuration of these mutations (Supplementary Table 2, Supplementary Fig. 2). We have also detected another secondary mutation in the post-progression biopsy in case 4 that is a known splice site mutation (c.4096+1G>A) downstream of the primary frameshift mutation. mRNA analysis has previously shown that this variant results in a shortened *BRCA1* isoform that lacks a large portion of exon 10, the *BRCA1*-Δ11q isoform, which would also splice out the primary mutation (27). This shortened isoform has been implicated as a potential mechanism of PARPi and platinum resistance (28).

In case 5 with a germline *RAD51C* mutation (c.577C>T, p.R193*), a post-progression biopsy of an enlarging groin lymph node contained four distinct secondary mutations that all restored the open reading frame of *RAD51C* (Table 1, Fig. 1a-d). The functional capacity of the primary mutation and four identified secondary mutations were investigated *in vitro* using the OVCAR8 OC cell line. Firstly, CRISPR/Cas9-mediated knockout (KO) of *RAD51C* resulted in increased cisplatin and rucaparib sensitivity that could be reverted to the level of parental cell resistance by introduction of wild-type *RAD51C* cDNA (Fig. 1e, Supplementary Fig. 3). Conversely, introduction of *RAD51C* cDNA with the primary mutation failed to convey resistance, whereas *RAD51C* cDNA containing the secondary mutations did confer resistance to rucaparib, as well as to multiple other PARPi (olaparib, niraparib, talazoparib and veliparib) and platinum compounds (cisplatin and carboplatin) (Fig. 1e, Supplementary Fig. 3, and Supplementary Table 3).

RAD51 foci formation assays confirmed proficient HR repair in cells complemented with wild-type *RAD51C* or any of the secondary *RAD51C* mutations tested, but not with the primary *RAD51C* mutation (Fig. 1f-g, Supplementary Fig. 4). Clear evidence for HR restoration was obtained using a standard HR reporter assay, in which a single genomic double-strand break is generated by the I-

SceI endonuclease to induce HR (Fig. 1h, Supplementary Fig. 5). *RAD51C*^{-/-} MCF10A cells had substantially reduced HR repair compared to wild-type cells or *RAD51C*^{-/-} cells expressing wild-type RAD51C. While the *RAD51C* primary mutation R193* failed to complement the HR defect of *RAD51C*^{-/-} cells, expression of all four *RAD51C* secondary mutants was able to restore HR comparable to wild-type *RAD51C*. We further demonstrated that *RAD51C* secondary mutants restore RAD51C R193* yeast-2-hybrid interactions with binding partners RAD51B and XRCC3 (Fig. 1i and Supplementary Fig. 6).

In order to assess the relative functional capacity of the *RAD51C* variants *in vivo*, a PDX was generated from the post-progression lymph node biopsy from case 5. Deep amplicon sequencing of *RAD51C* exon 4 was performed on two adjacent core biopsies of the lymph node, one of which had given rise to the PDX, and on tumors from three recipient-first passage (T1) mice (Fig. 2a). Whilst the first core contained all four *RAD51C* secondary mutations, only two of these were detected in the second core (Fig. 2b). Furthermore, microscopic spatial heterogeneity was observed between adjacent 40 µm sections within the second core (Supplementary Fig. 7). The portion of the second core biopsy used to generate the PDX predominately contained the c.574_577delinsGGCG mutation, as did PDX tumors from all three T1 mice, which had been implanted with tissue from the same core (Fig. 2a-b).

To understand the context of the heterogeneity further, SNP array analysis (archival tissue and post-progression biopsies) and whole-genome sequencing (WGS) of the post-progression biopsy and two T1 PDXs were performed. Copy number variation analysis revealed high levels of genomic instability in the archival sample and post-progression biopsies, with multiple amplifications and deletions detected throughout the genome. Little diversity was observed between the post-progression core biopsy that gave rise to the PDX, and the PDX itself; the two T1 PDX analyzed were also highly concordant (Fig. 2c-d, Supplementary Table 4). Three copies of the *RAD51C* gene

were observed in both archival and post-progression biopsies, which was confirmed by FISH analysis of post-progression and PDX tissue (Fig. 2e).

For case 6 with a germline *RAD51D* mutation (c.770_776del, p.G258Sfs*50), post-progression biopsy samples were collected from two different sites, a metastasis in the liver that was still responding to treatment and a growing metastasis in the spleen (Fig. 3a-c). The *RAD51D* secondary mutation (c.770_776delinsA) was found only in the splenic lesion that was progressing on rucaparib, suggesting that the detected secondary mutation conferred resistance (Table 1, Fig. 3d). To investigate the potential molecular basis of restored function observed with the *RAD51D* secondary mutation, we conducted molecular dynamics modeling of wild-type RAD51D, as well as RAD51D with both the primary and secondary mutations. Accurate simulation of the primary mutation was not possible because the frameshift-altered sequence diverged significantly from the sequence in the crystal structure. Molecular dynamics simulations indicated that in a DNA-RAD51D homo-filament the S-G-R residues, which are replaced with lysine in the secondary mutation, were involved in dsDNA binding (Supplementary Fig. 8a-b and Supplementary Video); and modeling of the RAD51D with the secondary mutation indicated that the interaction with dsDNA was maintained (Supplementary Fig. 8c). Arginine to lysine substitutions are tolerated in evolutionary comparisons of this particular residue (Supplementary Fig. 8d), the interaction with dsDNA suggests that this secondary mutation can confer partial restoration of function.

The functional capacity of the primary and secondary *RAD51D* mutations was further investigated *in vitro* using a previously described immortalized Chinese hamster (CHO) *RAD51D* KO cell line (29) and HR-competent human high-grade serous OC cell line PEO4 (containing functional *BRCA2*). Introduction of *RAD51D* cDNA with the secondary mutation (c.770_776delinsA) conferred resistance to cisplatin, rucaparib and other PARPis, while *RAD51D* cDNA containing the primary mutation did not (Fig. 3e, Supplementary Fig. 9). Two PEO4-derived clones, which

contained either a homozygous frameshift mutation (c.762_763del, p.D254Efs*72) in the same exon as the primary frameshift mutation, or the homozygous secondary mutation (c.770_776delinsA, p.S257_R259delinsK) in endogenous *RAD51D*, were generated using CRISPR homology-directed repair. PEO4 with the frameshift mutation had increased cisplatin and rucaparib sensitivity compared with parental PEO4 or the cells with the secondary mutation (Fig. 3f, Supplementary Fig. 10). Furthermore, RAD51 foci formation assay confirmed proficient HR repair in parental PEO4 and cells expressing the secondary mutation, but not in cells with the frameshift mutation (Fig. 3g).

Discussion

In order to understand the development of secondary resistance to PARPi therapy, we analysed 12 cases in which paired tumor biopsies were obtained both before treatment and following tumor progression, from patients with high-grade OC receiving the PARPi rucaparib on the ARIEL2 Part 1 trial. Six of twelve cases were found to contain mutations in one of three DNA repair genes, *BRCA1*, *RAD51C* or *RAD51D*, prior to therapy. Strikingly, in five of these six cases at progression we identified secondary mutations that restored the open reading frame by next-generation sequencing of progressing lesions, including two cases that had two or more secondary mutations. In a seventh case, we detected a somatic frameshift mutation in *CDK12*, a reported regulator of HR, although no secondary mutations were observed in the progressing lesion.

Since secondary mutations in *RAD51C* and *RAD51D* have not been previously described in preclinical or clinical studies, we focused in detail on those mutations. Four distinct *RAD51C* secondary mutations were identified within one core biopsy, highlighting the selective pressure for the tumor cells to restore HR repair in the face of PARPi treatment. The observed microscopic spatial heterogeneity in the relapsed lymph node extends a previous report by Patch *et al.* (14), where 12 distinct secondary *BRCA2* events were detected within one autopsy case, including five events, each identified at more than one metastatic site.

Functional analyses demonstrated that the *RAD51C* cDNA with the secondary mutations were able to restore RAD51C function in *RAD51C* KO ovarian cancer cells, as evidenced by increased homologous recombination, restored ability to bind the RAD51C binding partners RAD51B and XRCC3, and increased resistance to platinum and PARP inhibitors. Based on the observed variant frequencies reported for this case, including FISH and WGS analyses, we predict that each primary cancer cell contained three copies of the primary *RAD51C* mutation, whereas each cell with acquired resistance contained two copies of the primary mutation and one of four possible

secondary mutations (Supplementary Fig. 11). We generated a PDX from the post-progression biopsy containing the *RAD51C* secondary mutations. However, with only one secondary mutation predominant in the biopsy material from which the PDX was generated and in the PDX itself, it was not possible to detect relative functional selection of the four secondary *RAD51C* mutations present in that lymph node under rucaparib pressure *in vivo*.

Molecular dynamics modeling of the RAD51D species with the secondary mutation revealed that Ser257-Gly258-Arg259 to Lys substitution maintains the interaction with dsDNA, which was observed in the wild type RAD51D model. This was further supported by the presence of lysine at codon 259 in non-human RAD51D, suggesting that this secondary mutation could confer partial or full restoration of function. *In vitro* functional analyses of the primary and secondary *RAD51D* mutations provided additional evidence that the primary mutation sensitized cells to platinum and PARPi compounds, whilst the secondary mutation conferred resistance to both.

These observations provide evidence for restoration of functional HR under PARPi selection pressure in tumors with *RAD51C* or *RAD51D* mutations, supporting the view that mutations in these genes are synthetically lethal with PARP inhibition and demonstrating that secondary mutations are an important clinical mechanism of resistance in non-*BRCA1/2* HR genes. Moreover, we observed secondary mutations at progression following PARPi therapy in five of six cases containing a primary mutation in a DNA repair gene at diagnosis. Collectively, these results identify the need for sequencing PARPi therapy early during a patient's disease course and highlight the urgent need for development of PARPi-containing combination or sequencing strategies capable of more robust cell killing, in order to circumvent or delay the development of PARPi resistance

In summary, *in vitro* and *in vivo* analyses of primary and secondary mutations in *RAD51C* and *RAD51D* provided evidence for secondary mutations restoring most of the open reading frame beyond the primary mutation, thereby re-instituting HR function and contributing to development of clinical resistance to the PARPi rucaparib. Furthermore, these data support the role of primary mutations of *RAD51C* and *RAD51D* in conferring PARP inhibitor sensitivity and reveal secondary mutations in these genes as a mechanism of acquired PARPi resistance.

Methods

Patient samples

Archival tumor and a tumor deposit suitable for attempted pre-treatment biopsy were required for all patients who enrolled in the ARIEL2 Part 1 trial (NCT01891344). A post-progression tumor biopsy was optional. RECIST imaging & CA-125 level monitoring was recorded for the duration of patients' enrolment in the trial.

Cell lines and Culture

The human ovarian carcinoma cell line OVCAR8 was obtained from National Cancer Institute (Rockville, MD). Early passages of the parental OVCAR8 and *RAD51C* KO 2-130 were banked, tested for *Mycoplasma* and STR profiled; subsequent thaws were used within 6 months. The PEO4 cell line was obtained from F. Couch, Mayo Clinic in 2013 and viably stored until 2016; subsequent thaws were used within 6 months. The PEO4 cells were routinely tested for *Mycoplasma* and were last authenticated by STR profiling in April 2017. The CHO cell lines parental and deficient for *RAD51D* were obtained from Drs. Larry Thompson and Claudia Wiese, tested for *Mycoplasma*, and passaged for 2 months. The MCF10A cells were provided by B.H. Park (John Hopkins University School of Medicine). Early passage cells obtained were integrated with DR-GFP reporter and viably stored; subsequent thaws were used within 4 months. These modified cells were used for generating *RAD51C* conditional mutants and subsequent experiments. *RAD51C* conditional MCF10A cell line was tested negative for *Mycoplasma* on May 10th, 2017 using the MycoAlert PLUS assay kit from Lonza.

The OVCAR8 cell line was cultured in RPMI 1640 (Corning) supplemented with 10% fetal bovine serum (Peak Serum) and 1% penicillin and streptomycin (Corning) in a 5% CO₂ atmosphere at 37°C. The CHO cell line was cultured in MEM alpha (Corning) with 10% FBS (Peak) and 1% P/S (Corning) in 5% CO₂ at 37 °C. The PEO4 and OVCAR8 cell lines (for RAD51 foci formation

assays) were cultured in DMEM/F-12, GlutaMAX™ Supplement medium (Gibco™) containing 5 µg/ml insulin, 50 ng/ml EGF and 1 µg/ml hydrocortisone in a 5% CO₂ atmosphere at 37°C. The MCF10A cell line was cultured in DMEM HG/F-12 supplemented with 5% horse serum, 1% penicillin and streptomycin, 100 ng/ml cholera toxin, 20 ng/ml epidermal growth factor, 0.01 mg/ml insulin and 500 ng/ml hydrocortisone in a 5% CO₂ atmosphere at 37 °C.

Compounds

Rucaparib camsylate salt was manufactured by Lonza (Basel, Switzerland). Carboplatin, cisplatin, olaparib, niraparib, talazoparib, and veliparib were obtained from MedChem Express.

Generation of OVCAR8 KO (clone 2-130) and overexpressing cell lines

To generate OVCAR8 KO (clone 2-130), OVCAR8 cells were seeded at 0.2×10^6 cells per well in 6-well plates on day 1 in complete media. On day 2, cells were transfected with Eugene 6 (Promega) according to the manufacturer's recommendation with 1 µg RAD51C CRISPR plasmid (Santa Cruz Biotechnology). Transfected cells were selected with 2 µg/ml puromycin (Thermo Fisher Scientific). Cells were single cell cloned by limiting dilution, and expanded in complete media without puromycin. RAD51C KO was confirmed by genomic DNA sequencing. DNA was isolated using PureLink Genomic DNA mini kit (Thermo Fisher Scientific), and PCR was performed using KOD Hot Start Master Mix (EMD Millipore) with the follow primers: forward primer 5'-gcagaagccttagaaactctgc and reverse primer 5'-tgaataacgcagaaacttcctg, according to the manufacturer's recommendation. The PCR product was purified using a QIAquick PCR Purification kit (Qiagen) and Sanger sequencing was performed with primer 5'-tttcattaagggcactccacc. RAD51C clone 2-130 sequences showed 35 bp deletion *RAD51C* c.231_264 that generated nonsense mutation p.E80*.

To generate cells that transiently express wild-type and mutated RAD51C for RAD51 foci analysis, cells were transfected with commercially obtained, mutated *RAD51C* pcDNA3.1(+)/Hygro plasmids (OHu21400C, GenScript) using Lipofectamine™ 3000 Reagent (Thermo Fisher Scientific), following manufacturer's instructions. After 48 hour recovery period transfected cells were selected with 200 µg/ml hygromycin (Thermo Fisher Scientific). To generate stable overexpressing *RAD51C* mutants, gene fragments containing specific mutations were synthesized (Integrated DNA Technologies) and cloned into the lentivector encoding RAD51C open reading frame under the control of the PGK promoter (Genecopoeia). Lentivirus vectors were packaged in HEK293 cells with 3rd generation lentiviral system (System Biosciences), and RAD51C 2-130 cells were transduced with 15 MOI plus polybrene 8 µg/ml for 24 hours. Cells were cultured in complete media for 48 hours before puromycin selection.

Generation of mutant *RAD51D* overexpressing CHO *RAD51D* KO cell lines

To generate stable overexpressing *RAD51D* mutants, gene fragments containing specific mutations were synthesized (Integrated DNA Technologies) and cloned into the lentivector encoding *RAD51D* open reading frame (NM_002878.3) under the control of the PGK promoter (Genecopoeia). Lentivirus vectors were packaged in HEK293 cells with 3rd generation lentiviral system (System Biosciences), and CHO *RAD51D* KO cells (29) were transduced with 15 MOI plus polybrene 8 µg/ml for 24 hours. Cells were cultured in complete media for 48 hours before puromycin selection.

Generation of PEO4 cell line with the secondary *RAD51D* mutation

To generate PEO4 cells with the secondary *RAD51D* mutation (c.770_776delinsA), PEO4 cells were transduced with lentiviral Cas9 vector (PUCas9Cherry) and doxycycline-inducible CRISPR guide with GFP vector (FgH1tUTG) with CRISPR guide 5'-CAACCACATAACTCGAGACA (30). 40 pmol of ssODN containing the secondary mutation and a silent PAM mutation with 80 bp sequence overlap on each side (IDT) was transfected using Lipofectamine™ 3000 Reagent (Thermo

Fisher Scientific), following the manufacturer's instructions. After overnight incubation, CRISPR guide was activated by doxycycline-supplemented medium (1 µg/ml) for 3 days. mCherry and GFP double-positive cells were single-cell plated using flow cytometry. After expanding for at least two weeks, single cell colonies were sequenced for the presence of homozygous secondary mutation using MiSeq platform. The colony with the frameshift *RAD51D* mutation (c.762_763del, p.D254Efs*72) was also selected from this process.

RAD51 foci formation assay

Cells were treated with DMSO or 10 µM rucaparib for 24, 48 or 72 hours. Cells were fixed with either 4% paraformaldehyde or methanol, permeabilised with 0.2% TritonX-100, blocked with blocking buffer (1% BSA, 2% FCS or 1% BSA, 3% milk, 2% goat serum in PBS) and incubated with rabbit anti-RAD51 (Abcam) and either mouse anti-Geminin (Abcam) or mouse anti- γ H2AX (Abcam) antibodies. For RAD51 foci formation with geminin staining, anti-rabbit 647 and anti-mouse 546 Alexa Fluor[®] secondary antibodies (Invitrogen, Molecular Probes[™]) were used. Nuclei were counterstained with Hoechst 33342 (NucBlue[®] Live ReadyProbes[®] Reagent; Thermofisher Scientific). Cells were imaged using a LSM 780 inverse laser scanning microscope (Zeiss) and captured with a LSM T-PMT detector (Zeiss). At least 194 cells from 4 fields of view and 2 independent experiments were counted. Cells with ≥ 5 RAD51 foci/nucleus were scored using CellProfiler (version 2.2.0, Broad Institute). For RAD51 and γ H2AX foci formation assay, anti-rabbit 488 and anti-mouse 594 Alexa Fluor[®] secondary antibodies (Invitrogen, Molecular Probes[™]) were used. Nuclei were counterstained with DAPI (Sigma). Cells were imaged using Leica DM 1000 LED at 40x.

Cell Viability Assays

Endpoint viability assays were performed using the CellTitre-Glo (Promega) assay according to the manufacturer's protocol. Briefly, cells were seeded at 600-800 cells per in 384-well plates or 2000

cells in 96-well plates, and allowed to establish overnight before adding treatments. Cells were treated for 6-7 days with compounds, over a range of concentrations, then assay was terminated and viability assessed using luminescence detection on a Victor X4 plate reader (Perkin Elmer). Luminescence was normalized to DMSO control, and IC₅₀ values were calculated using a sigmoidal dose-response curve fit analysis (Prism software, GraphPad).

HR reporter assay

The DR-GFP reporter was introduced into MCF10A cells as previously described (31). Cre recombinase was expressed in conditional RAD51C^{-/-} MCF10A cells to remove an ectopic floxed RAD51C gene (R.P. and M.J., unpublished) and in isogenic wild-type cells as a control. Post-Cre expression, cells were infected with an I-SceI-expressing lentivirus. GFP⁺ cells were measured by flow cytometry (BD FACScan) 48 hours after infection and data were analyzed using FlowJo software. Without I-SceI expression the number of GFP⁺ cells was ≤ 0.01 .

Creation of Y2H and pWZL expression vectors

The RAD51C mutants were generated in the yeast-two-hybrid (Y2H) plasmids (pGAD-C1 and pGBD-C1) and pWZL plasmid using site-directed mutagenesis (Supplementary Table 5). RAD51C and RAD51B cDNA were sub-cloned into the pGAD-C1 and pGBD-C1 vectors using 5'-EcoRI and 3'-SalI restriction sites. The RAD51C and RAD51B cDNA was a gift from Jun Huang (32) and the pGAD-XRCC3 and pGBT-XRCC3 plasmids were a gift from David Schild (33).

Y2H assay

The yeast-2-hybrid experiments were performed as previously described (34), except that the indicated *GAL4* activating domain- and binding domain-expressing vectors were co-transformed into the YPJ694a yeast strain.

BRCA1 colony PCR

10 ng genomic DNA from post-progression biopsy (case 4) was PCR amplified with primers: forward 5'-gcatgtcgacGGGAACCTAACCAAACGGAGCA and reverse 5'-atgcaagcttGAGATCTTTGGGG-TCTTCAGCA. Primers were designed with restriction sites for SalI and HindIII. Reaction was performed with KOD polymerase (EMD Millipore) according to manufacturer's recommendation, and cleaned by QIAquick PCR kit (Qiagen). pUC19 (NEB) and PCR product were cut with SalI (NEB) and HindIII (NEB), and cleaned by QIAquick PCR kit, before ligating with T4 ligase (NEB) and transforming *E. coli* bacteria. Colonies were amplified by rolling circle amplification using bacteriophage phi29 DNA polymerase (NEB), before Sanger sequencing with M13 Forward-20 primer.

Western Blotting – OVCAR8, CHO and PEO4

Whole cell lysates were prepared using RIPA buffer (Sigma) containing protease inhibitor cocktail and protein concentration was assessed by BCA colourimetric protein determination (Pierce). Equal protein loads were resolved on precast 4–12% Bis-Tris gels under reducing conditions. Protein was transferred to nitrocellulose membranes using iBlot dry transfer method (Invitrogen), then probed with primary antibody anti-RAD51C (Santa Cruz Biotechnology), anti-RAD51D n-terminal (Abcam), anti-RAD51D c-terminal (Santa Cruz Bio), anti-GAPDH (Cell Signaling), anti-tubulin (Cell Signaling) or anti-actin (Abcam) followed by peroxidase-labelled secondary antibody and visualised by enhanced chemiluminescence (SuperSignal Chemiluminescent Substrate; Thermo Scientific) or IRDye-labelled secondary antibody (LI-COR Biotechnology) according to the manufacturer's instructions. Band volume analysis was conducted using Odyssey Fc (LI-COR Biotechnology).

Western Blotting – MCF10A (subcellular)

Nuclear extracts were collected from MCF10A cells using the cytoplasmic lysis buffer (10 mM Tris-HCl, 0.34 M sucrose, 3 mM CaCl₂, 2 mM Magnesium acetate, 0.1 mM EDTA, 0.5% NP-40, protease inhibitor) and nuclear lysis buffer, (20 mM HEPES, 3 mM EDTA, 10% glycerol, 150 mM potassium acetate, 1.5 mM MgCl₂, 0.1% NP-40, protease inhibitor). 30 µg of nuclear protein was used for detection. Protein was separated on 10% acrylamide gels and transferred to PVDF membranes, and protein was detected on a LiCor CLX scanner. RAD51C expression was detected with RAD51C antibody (ab55728 1:500; Abcam), and equal nuclear loading was detected using PCNA antibody (sc-56 1:250; SantaCruz), and IR dye secondary antibodies from LiCor Biosciences. The image was adjusted for brightness and contrast using Photoshop (Adobe Systems Incorporated).

Western Blotting –Y2H

Yeast expressing the indicated AD and BD constructs were grown overnight at 30°C in 5 ml YPD and then diluted to 0.2 OD₆₀₀ in YPD for 90 minutes. Whole cell lysates of equal cell numbers (0.2 OD₆₀₀) was extracted by TCA precipitation. Protein was separated on 10% acrylamide gels and transferred to PVDF membranes, and protein was detected on a LiCor CLX scanner. RAD51C expression was detected with RAD51C antibody (ab55728 1:500; Abcam), equal loading was detected using a Kar2 antibody (sc-33630 1:2000; Santa Cruz), and IR dye secondary antibodies (1:20,000) from LiCor Biosciences. The image was adjusted for brightness and contrast using Photoshop (Adobe Systems Incorporated).

Generation and treatment of patient-derived xenografts (PDX)

All experiments involving animals was approved by the Walter and Eliza Hall Institute of Medical Research Animal Ethics Committee. A patient-derived xenograft (PDX) was generated from the post-progression lymph node biopsy by transplanting fresh fragments subcutaneously into six NOD/SCID IL2R γ null recipient mice (T1 – passage 1; ref. 35), three of which developed tumors. The PDX tumors generated were transplanted into recipient mice (T2 – passage 2), minced and cryopreserved in DMSO and snap frozen for further analysis. A mouse harboring T2 PDX tumor was treated with 450 mg/kg rucaparib for 2 weeks (oral gavage once daily Monday-Friday) at ~0.1 cm³ in size. The tumor was harvested and snap frozen 24 hours after treatment completion. DNA was extracted from the archival tumor sample and from the post-progression biopsy for sequencing analysis. SNP array analysis was performed on archival tissue and the post-progression biopsy and WGS was performed on the post-progression biopsy and two T1 PDXs.

Serial Section Analysis

A small fragment of one of the snap-frozen post-progression biopsy cores from patient 5 with the germline *RAD51C* mutation was embedded in Tissue-Tek[®] O.C.T. compound (Sakura Finetek). Six serial sections were collected (40 μ m each) with a 4 μ m section cut for H&E staining in between each section. Direct PCR was performed on each serial section using Phusion Human Specimen Direct PCR Kit (Thermo Scientific[™]) following manufacturer's instructions. Briefly, 25 μ l of buffer and 0.5 μ l of DNA release reagent was added to each tube with section scrolls and incubated at 98C for 2 mins. Amplicon libraries were prepared using a two-step PCR approach: first internal PCR to amplify the region of interest (5'-tcgtcggcagcgtcagatgtgtataagagacag-tccaaaggagaacattttgtta forward primer and 5'-gtctcgtgggctcggagatgtgtataagagacag-tgtgtagtcacgacagcgaaa reverse primer), followed by second outer PCR to add the sequencing adaptors and indexes for multiplexing). Briefly, 2 μ l of digested section sample for first PCR or inner product for second PCR was added to 5 μ l of 5 \times Q5 Reaction buffer (NEB), 1 μ l of each primer (forward and reverse; 2nM each) for first PCR or 1 μ l of each Nextera XT index (unique i5 and i7, Illumina), 0.5 μ l of dNTPs (10 mM each),

0.25 µl of Q5 Hot Start High-Fidelity DNA Polymerase (NEB), and 15.25 µl of dH₂O. The PCR was performed as follows: 30 seconds at 98 °C for initial denaturation, followed by 20 cycles of 10 seconds at 98 °C, 15 seconds at 60 °C for first PCR or 63°C for second PCR, and 20 seconds at 72 °C, followed 2 minutes at 72 °C for final extension. The libraries were cleaned using standard Agencourt AmpureXP beads (Beckman Coulter) procedure with DNA to bead ratio of 1:0.9, and normalised to 1 nM concentration (using the Qubit dsDNA HS Assay Kit for quantification). Libraries were sequenced using MiSeq Nano v2 300-cycle kit (Illumina) at 10 pM final concentration to a minimum depth of 30,000x. Reads were aligned using bwa-mem to the Human GRCh38, and visualized for further analysis using IGV browser (36).

Genomic analysis

All tumors were sequenced using Foundation Medicine's NGS-based assay (19). Analysed data were plotted using OncoPrint. Germline HRR gene mutations were confirmed by sequencing of DNA extracted from blood using the NGS-based BROCA assay (37).

Whole-genome sequencing libraries were prepared for a post-progression biopsy of patient 5 and the two first-passage PDX tumors generated from this biopsy. The libraries were prepared using the TruSeq Nano library preparation kit (Illumina), and the sequencing was performed on the Illumina X Ten platform (Kinghorn Centre for Clinical Genomics, Darlinghurst).

Adaptor sequences were removed with Trimmomatic 0.36 (38) before mapping to the Human GRCh38 (GCA_000001405.15) and Mouse GRCm38(GCF_000001635.25) genomes with Bowtie2 2.2.5 (39). Human reads were separated from mouse background with Xenomapper (40). Reads were sorted and indexed with Samtools 1.3.1 (41). Copy number analysis was performed with HMMcopy 1.16 (42) and plotted with CIRCOS 0.67 (43). Coriell Cell Repository NA12878 reference cell line DNA previously processed with the TruSeq Nano kit at KCCG was used as an unrelated normal control.

High-depth amplicon analysis of *RAD51C* exon 4 was performed on the multiple samples from patient 5 with germline *RAD51C* mutation (pre-treatment biopsy, post-progression biopsy and 4 PDX tumors) in triplicate. DNA was extracted using QIAamp DNA mini kit (QIAGEN). Amplicon libraries were prepared using a two-step PCR approach, as described for serial section analysis, with 20 ng DNA input in the first PCR. The PCR was performed as follows: 30 seconds at 98 °C for initial denaturation, followed by 16 cycles for first PCR or 12 cycles for second PCR of 10 seconds at 98 °C, 15 seconds at 60 °C for first PCR or 63°C for second PCR, and 20 seconds at 72 °C, followed 2 minutes at 72 °C for final extension. The libraries were cleaned and sequenced as described for serial section analysis to a minimum depth of 10,000x. Reads were aligned using bwa-mem to the Human GRCh38, and visualized for further analysis using IGV browser (36).

***RAD51C* FISH**

Frozen OCT sections from the post-progression biopsy (Core 2 OCT block for serial sectioning) and the PDX sample treated with 450 mg/kg for 2 weeks, both from patient 5 with the germline *RAD51C* mutation, as well as an unrelated control PDX were thawed, fixed in 4% PFA and pre-treated with SPOT-Light[®] Tissue Pre-treatment kit (Invitrogen). Briefly, sections were incubated in pre-treatment solution for 15 minutes at 95°C, washed in PBS and incubated with enzyme for 10 minutes at room temperature. Sections were dehydrated, incubated with denaturation buffer (70% formamide, 2x SSC, pH 7.0-8.0) for 5 minutes at 73°C, dehydrated, and incubated with prepared *RAD51C* probe (as per manufacturer's instructions; Empire Genomics) for 24 hours at 37°C. Sections were then washed with WS1 (0.4X SSC/0.3% NP40) for 2 minutes at 73°C, followed by a wash with WS2 (2X SSC/0.1% NP40) for 1 minute at room temperature. Nuclei were counterstained with Hoechst 33342 (NucBlue[®] Live ReadyProbes[®] Reagent; ThermoFisher Scientific) and coverslipped with fluoromount G (SouthernBiotech). Sections were imaged using a LSM 780 inverse laser scanning microscope (Zeiss) and captured with a LSM T-PMT detector (Zeiss).

Molecular Dynamics Modeling

Molecular models were made of RAD51D to test the effects of deletions and mutations using NAMD (44). Initial models were constructed from primary sequence using the Swiss-Model web Server (45), which constructed a model using the *Saccharomyces cerevisiae* 1SZP pdb structure as a template (46). The model was constructed as a 6 monomer helical complex with an additional 58 base pair double stranded DNA loosely positioned along the central helical axis. A homomeric model was constructed as appropriate crystal structures were not available to allow modeling of the heteromeric RAD51B/C/D and XRCC2 complex. This model provides general information on interaction between the sub-units and between the protein and DNA. RAD51 paralogues and RecA have been shown to bind both ssDNA in an ATP catalyzed reaction and dsDNA in a filament structure. As a computational simplification, we chose to model only the interaction with dsDNA. A wild-type and two mutant models were constructed (p.Ser257_Arg259delinsLys and p.Ser257_Arg259fs*50, see Fig. 3d for annotation) using VMD (47) and the psfgen module. All models were solvated and ionized with sodium chloride to approximately 0.15M and electrical neutrality. The initial dimensions of the wild-type system were 108×108×200 Angstroms, with a total of 222339 atoms for the WT, and 230572 or 226326 atoms for the primary and secondary mutants, respectively. Each model was equilibrated for 1 nanosecond before performing production runs. Production runs were performed using NAMD 2.10 at 310K using a NPT ensemble (Constant pressure and temperature). Long-range Coulomb forces were computed with the Particle Mesh Ewald method with a grid spacing of 1 Å. 2 fs timesteps were used with non-bonded interactions calculated every 2 fs and full electrostatics every 4 fs while hydrogens were constrained with the SHAKE algorithm. The cut-off distance was 12 Å with a switching distance of 10 Å and a pair-list distance of 14 Å. Pressure was controlled to 1 atmosphere using the Nosé-Hoover Langevin piston method employing a piston period of 100 fs and a piston decay of 50 fs. Trajectory frames were captured every 100 picoseconds. Simulation trajectories were viewed with VMD (47).

Acknowledgements

We thank D. Schild for XRCC3 Y2H plasmids, J. Huang for the cDNA used to make the RAD51C and RAD51B Y2H vectors, Drs. Larry Thompson and Claudia Wiese for their generous gift of the RAD51D CHO cell lines, Dr. Marco Herald for the Cas9/CRISPR vectors and T. Phuong and E. Lieschke for technical assistance. This work was supported by fellowships and grants from the National Health and Medical Research Council (NHMRC Australia) Project grant 1062702 (CLS); the Cancer Council Victoria (Sir Edward Dunlop Fellowship in Cancer Research to CLS); the Victorian Cancer Agency (Clinical Fellowships to CLS CRF10-20, CRF16014); CRC for Cancer Therapeutics (PhD top-up scholarship to GH); the Stafford Fox Medical Research Foundation including a WEHI Centenary Stafford Fox Fellowship to HB; the National Institute of Health (2P50CA083636) (EMS) and the Wendy Feuer Ovarian Cancer Research Fund (EMS). This work was made possible through the Australian Cancer Research Foundation, the Victorian State Government Operational Infrastructure Support and Australian Government NHMRC IRIISS. We thank Clovis Oncology for funding Foundation Medicine analyses and providing rucaparib for in vivo experiments. We acknowledge Elaina Mann and Lan-Thanh Vo (Clovis Oncology) for tumor specimen shipment and processing. We thank Kyle Gowen and Joel Greenbowe (Foundation Medicine) for assistance in modeling of tumor clonal fractions with secondary RAD51C mutations. K.A.B. is supported by the National Institutes of Health Grant (ES024872), the V Foundation for Cancer Research, and a Stand Up to Cancer Innovative Research Grant (Grant Number SU2C-AACR-IRG-02-16). Stand Up To Cancer is a program of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the Scientific Partner of SU2C. R.P. was supported by NIH F32GM110978. M.J. is supported by NIH R01CA185660 and the MSK Cancer Center Support Grant/Core Grant (NIH P30CA008748). E.M.S., E.M.S., M.J., R.L.C. and S.H.K. are supported by a Stand Up To Cancer-Ovarian Cancer Research Fund-Ovarian Cancer National Alliance-National Ovarian Cancer Coalition Dream Team Translational Research Grant (Grant Number: SU2C-AACR-DT16-15). Stand Up To Cancer is a

program of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the Scientific Partner of SU2C. MD modelling was supported by Victorian Life Sciences Computation Initiative grant LTU0011 on its Peak Computing Facility at the University of Melbourne, an initiative of the Victorian Government, Australia. The Australian Ovarian Cancer Study was supported by the U.S. Army Medical Research and Materiel Command under DAMD17-01-1-0729, The Cancer Council Tasmania and The Cancer Foundation of Western Australia and the National Health and Medical Research Council of Australia (NHMRC; ID400413; ID400281).

The management group of the AOCS group included D Bowtell, G Chenevix-Trench, A Green, P Webb, A DeFazio and D Gertig. The project and data managers included N Traficante, S Fereday, S Moore, J Hung, K Harrap, T Sadkowsky, N Pandeya. The Research Nurses and Assistants of the AOCS group included: M Malt, A Mellon, R Robertson, T Vanden Bergh, M Jones, P Mackenzie, J Maidens, K Nattress, YE Chiew, A Stenlake, H Sullivan, B Alexander, P Ashover, S Brown, T Corrish, L Green, L Jackman, K Ferguson, K Martin, A Martyn, B Ranieri, J White, V Jayde, P Mamers, L Bowes, L Galletta, D Giles, J Hendley, K Alsop, T Schmidt, H Shirley, C Ball, C Young, S Viduka, Hoa Tran, Sanela Bilic, Lydia Glavinas, Julia Brooks. The Clinical and Scientific Collaborators of the AOCS group included: R Stuart-Harris, F Kirsten, J Rutovitz, P Clingan, A Glasgow, A Proietto, S Braye, G Otton, J Shannon, T Bonaventura, J Stewart, S Begbie M Friedlander D Bell, S Baron-Hay, A Ferrier (dec.), G Gard, D Nevell, N Pavlakis, S Valmadre, B Young, C Camaris, R Crouch, L Edwards, N Hacker, D Marsden, G Robertson, P Beale, J Beith, J Carter, C Dalrymple, R Houghton, P Russell, M Links, J Grygiel, J Hill, A Brand, K Byth, R Jaworski, P Harnett, R Sharma, G Wain, B Ward, D Papadimos, A Crandon, M Cummings, K Horwood, A Obermair, L Perrin, D Wyld, J Nicklin, M Davy, MK Oehler, C Hall, T Dodd, T Healy, K Pittman, D Henderson, J Miller, J Pierdes, P Blomfield, D Challis, R McIntosh, A Parker, B Brown, R Rome, D Allen, P Grant, S Hyde, R Laurie, M Robbie, D Healy, T Jobling, T

Manolitsas, J McNealage, P Rogers, B Susil, E Sumithran, I Simpson, K Phillips, D Rischin, S Fox, D Johnson, S Lade, M Loughrey, N O'Callaghan, W Murray, P Waring, V Billson, J Pyman, D Neesham, M Quinn, C Underhill, R Bell, LF Ng, R Blum, V Ganju, I Hammond, Y Leung, A McCartney (dec.), M Buck, I Haviv, D Purdie, D Whiteman, N Zeps.

David Bowtell on behalf of the Australian Ovarian Cancer Study gratefully acknowledges the cooperation of the participating institutions in Australia and also acknowledges the contribution of the study nurses, research assistants and all clinical and scientific collaborators to the study. We would like to thank all of the women who participated in these research programs.

References

1. Scott CL, Swisher EM, Kaufmann SH. Poly (ADP-Ribose) Polymerase Inhibitors: Recent Advances and Future Development. *J Clin Oncol*. 2015;33:1397–406.
2. Curtin NJ. DNA repair dysregulation from cancer driver to therapeutic target. *Nat Rev Cancer*. 2012;12:801–17.
3. Konstantinopoulos PA, Ceccaldi R, Shapiro GI, D'Andrea AD. Homologous Recombination Deficiency: Exploiting the Fundamental Vulnerability of Ovarian Cancer. *Cancer Discov*. 2015;5:1137–54.
4. Pommier Y, O'Connor MJ, De Bono J. Laying a trap to kill cancer cells: PARP inhibitors and their mechanisms of action. *Sci Transl Med*. 2016;8:362ps17.
5. Lord CJ, Ashworth A. PARP inhibitors: Synthetic lethality in the clinic. *Science*. 2017;355:1152–8.
6. Alsop K, Fereday S, Meldrum C, deFazio A, Emmanuel C, George J, et al. BRCA mutation frequency and patterns of treatment response in BRCA mutation-positive women with ovarian cancer: a report from the Australian Ovarian Cancer Study Group. *J Clin Oncol*. 2012;30:2654–63.
7. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol*. 2014;15:852–61.
8. Mirza MR, Monk BJ, Herrstedt J, Oza AM, Mahner S, Redondo A, et al. Niraparib Maintenance Therapy in Platinum-Sensitive, Recurrent Ovarian Cancer. *N Engl J Med*. 2016;375:2154–64.
9. Swisher EM, Lin KK, Oza AM, Scott CL, Giordano H, Sun J, et al. Rucaparib in relapsed, platinum-sensitive high-grade ovarian carcinoma (ARIEL2 Part 1): an international, multicentre, open-label, phase 2 trial. *Lancet Oncol*. 2017;18:75–87.
10. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature*. 2008;451:1116–20.
11. Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA, et al. Resistance to therapy caused by intragenic deletion in BRCA2. *Nature*. 2008;451:1111–5.
12. Norquist B, Wurz KA, Pennil CC, Garcia R, Gross J, Sakai W, et al. Secondary Somatic Mutations Restoring BRCA1/2 Predict Chemotherapy Resistance in Hereditary Ovarian Carcinomas. *J Clin Oncol*. 2011;29:3008–15.
13. Stover EH, Konstantinopoulos PA, Matulonis UA, Swisher EM. Biomarkers of Response and Resistance to DNA Repair Targeted Therapies. *Clin Cancer Res*. 2016;22:5651–60.
14. Patch A-M, Christie EL, Etemadmoghadam D, Garsed DW, George J, Fereday S, et al. Whole-genome characterization of chemoresistant ovarian cancer. *Nature*. 2015;521:489–94.
15. Barber LJ, Sandhu S, Chen L, Campbell J, Kozarewa I, Fenwick K, et al. Secondary mutations in BRCA2 associated with clinical resistance to a PARP inhibitor. *J Pathol*. 2013;229:422–9.
16. Pennington KP, Walsh T, Harrell MI, Lee MK, Pennil CC, Rendi MH, et al. Germline and somatic mutations in homologous recombination genes predict platinum response and survival in ovarian, fallopian tube, and peritoneal carcinomas. *Clin Cancer Res*. 2014;20:764–75.
17. Cancer Genome Atlas Research Network. Integrated genomic analyses of ovarian carcinoma. *Nature*. 2011;474:609–15.
18. McCabe N, Turner NC, Lord CJ, Kluzek K, Bialkowska A, Swift S, et al. Deficiency in the repair of DNA damage by homologous recombination and sensitivity to poly(ADP-ribose) polymerase inhibition. *Cancer Res*. 2006;66:8109–15.

19. Frampton GM, Fichtenholtz A, Otto GA, Wang K, Downing SR, He J, et al. Development and validation of a clinical cancer genomic profiling test based on massively parallel DNA sequencing. *Nat Biotechnol.* 2013;31:1023–31.
20. Song H, Dicks E, Ramus SJ, Tyrer JP, Intermaggio MP, Hayward J, et al. Contribution of Germline Mutations in the RAD51B, RAD51C, and RAD51D Genes to Ovarian Cancer in the Population. *J Clin Oncol.* 2015;33:2901–7.
21. Prakash R, Zhang Y, Feng W, Jasin M. Homologous recombination and human health: the roles of BRCA1, BRCA2, and associated proteins. *Cold Spring Harb Perspect Biol* 2015;7:a016600.
22. Norquist BM, Harrell MI, Brady MF, Walsh T, Lee MK, Gulsuner S, et al. Inherited Mutations in Women With Ovarian Carcinoma. *JAMA Oncol.* 2016;2:482–90.
23. Meindl A, Hellebrand H, Wiek C, Erven V, Wappenschmidt B, Niederacher D, et al. Germline mutations in breast and ovarian cancer pedigrees establish RAD51C as a human cancer susceptibility gene. *Nat Genet.* 2010;42:410–4.
24. Loveday C, Turnbull C, Ramsay E, Hughes D, Ruark E, Frankum JR, et al. Germline mutations in RAD51D confer susceptibility to ovarian cancer. *Nat Genet.* 2011;43:879–82.
25. Joshi PM, Sutor SL, Huntoon CJ, Karnitz LM. Ovarian cancer-associated mutations disable catalytic activity of CDK12, a kinase that promotes homologous recombination repair and resistance to cisplatin and poly(ADP-ribose) polymerase inhibitors. *J Biol Chem.* 2014;289:9247–53.
26. Ekumi KM, Paculova H, Lenasi T, Pospichalova V, Bosken CA, Rybarikova J, et al. Ovarian carcinoma CDK12 mutations misregulate expression of DNA repair genes via deficient formation and function of the Cdk12/CycK complex. *Nucleic Acids Res.* 2015;43:2575–89.
27. Bonatti F, Pepe C, Tancredi M, Lombardi G, Aretini P, Sensi E, et al. RNA-based analysis of BRCA1 and BRCA2 gene alterations. *Cancer Genet Cytogenet.* 2006;170:93–101.
28. Wang Y, Bernhardt AJ, Cruz C, Krais JJ, Nacson J, Nicolas E, et al. The BRCA1- 11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Research.* 2016;76:2778–90.
29. Hinz JM, Tebbs RS, Wilson PF, Nham PB, Salazar EP, Nagasawa H, et al. Repression of mutagenesis by Rad51D-mediated homologous recombination. *Nucleic Acids Research.* 2006;34:1358–68.
30. Aubrey BJ, Kelly GL, Kueh AJ, Brennan MS, O'Connor L, Milla L, et al. An Inducible Lentiviral Guide RNA Platform Enables the Identification of Tumor-Essential Genes and Tumor-Promoting Mutations In Vivo. *CellReports.* The Authors; 2015;10:1422–32.
31. Pierce AJ, Johnson RD, Thompson LH, Jasin M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.* 1999;13:2633–8.
32. Liu T, Wan L, Wu Y, Chen J, Huang J. hSWS1•SWSAP1 is an evolutionarily conserved complex required for efficient homologous recombination repair. *J Biol Chem.* 2011;286:41758–66.
33. Schild D, Lio YC, Collins DW, Tsomondo T, Chen DJ. Evidence for simultaneous protein interactions between human Rad51 paralogs. *J Biol Chem.* 2000;275:16443–9.
34. Godin SK, Meslin C, Kabbinavar F, Bratton-Palmer DS, Hornack C, Mihalevic MJ, et al. Evolutionary and functional analysis of the invariant SWIM domain in the conserved Shu2/SWS1 protein family from *Saccharomyces cerevisiae* to *Homo sapiens*. *Genetics.* 2015;199:1023–33.
35. Topp MD, Hartley L, Cook M, Heong V, Boehm E, McShane L, et al. Molecular correlates of platinum response in human high-grade serous ovarian cancer patient-derived xenografts. *Mol Oncol.* 2014;8:656–68.
36. Robinson JT, Thorvaldsdottir H, Winckler W, Guttman M, Lander ES, Getz G, et al. Integrative genomics viewer. *Nat Biotechnol.* 2011;29:24–6.

37. Walsh T, Casadei S, Lee MK, Pennil CC, Nord AS, Thornton AM, et al. Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc Natl Acad Sci*. 2011;108:18032–7.
38. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9:357–9.
39. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics*. 2014;30:2114–20.
40. J Wakefield M. Xenomapper: Mapping reads in a mixed species context. *JOSS*. 2016;1:18.
41. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25:2078–9.
42. Ha G, Roth A, Lai D, Bashashati A, Ding J, Goya R, et al. Integrative analysis of genome-wide loss of heterozygosity and monoallelic expression at nucleotide resolution reveals disrupted pathways in triple-negative breast cancer. *Genome Res*. 2012;22:1995–2007.
43. Krzywinski M, Schein J, Birol I, Connors J, Gascoyne R, Horsman D, et al. Circos: an information aesthetic for comparative genomics. *Genome Research*. 2009;19:1639–45.
44. Phillips JC, Braun R, Wang W, Gumbart J, Tajkhorshid E, Villa E, et al. Scalable molecular dynamics with NAMD. *J Comput Chem*. 2005;26:1781–802.
45. Bordoli L, Kiefer F, Arnold K, Benkert P, Battey J, Schwede T. Protein structure homology modeling using SWISS-MODEL workspace. *Nat Protocols*. 2009;4:1–13.
46. Conway AB, Lynch TW, Zhang Y, Fortin GS, Fung CW, Symington LS, et al. Crystal structure of a Rad51 filament. *Nat Struct Mol Biol*. 2004;11:791–6.
47. Humphrey W, Dalke A, Schulten K. VMD: visual molecular dynamics. *J Mol Graph*. 1996;14:33–8–27–8.

Table Legends

Table 1. Clinical and molecular features of twelve OC cases with post-progression biopsies.

The number of prior chemotherapeutic regimens, best response to treatment on ARIEL2 Part 1, progression free survival (PFS) on ARIEL2 Part 1 and details of the mutations detected in known Homologous Recombination (HR) DNA repair genes are shown. Seven of twelve cases were found to contain a mutation in either an HR gene (*BRCA1* or *RAD51C/D*) or a gene that impacts transcription of multiple HR genes (*CDK12*), five of which contained secondary mutations in the post-progression biopsy. For five cases, no HR or related gene mutation was observed in archival, pre-treatment or post-progression tumor material. Secondary mutations were only identified in post-progression cases with HR gene mutations (p=0.015, Fisher's exact). Number of prior regimens, best response by RECIST/GCIG CA-125 criteria. RECIST, CA-125, and PFS data based on the data cut-off date of April 29, 2016. LOH status was determined from variant allele frequency. Transcripts used for variant annotation: *BRCA1*, NM_007294.3; *RAD51C*, NM_058216.2; *RAD51D*, NM_002878.3; and *CDK12*, NM_016507.3. CA-125 not evaluable (NE); germline mutation (GL); somatic mutation (SOM); secondary mutation (2° MUT); loss of heterozygosity (LOH). # Tumor purity was estimated based on the genome-wide copy number analysis by Foundation Medicine. ^The reported *RAD51C* mutation frequencies were estimated from biopsy core 1 using Foundation Medicine's T5 assay. To investigate tumor heterogeneity in more detail this sample was sequenced using a single amplicon assay (see Fig. 2). †*RAD51D* secondary mutation was only detected in the non-responding splenic lesion, while only the primary mutation was detected in the responding liver lesion (see Supp. Table 1).

Figure Legends

Figure 1. Identification and functional assessment of *RAD51C* secondary mutations identified in the post-progression biopsy in case 5. **a**, RECIST measurements of three metastatic sites, which were monitored in the patient identified to have a germline *RAD51C* mutation (c.577C>T). None of these lesions progressed during rucaparib treatment. After 11 months of rucaparib treatment the patient developed a new enlarged groin lymph node, which was biopsied. **b**, Serum CA-125 levels monitored during the ARIEL2 Part 1 trial in the patient with the germline *RAD51C* mutation (c.577C>T). **c**, CT scans prior to, during, and following treatment of the patient with the germline *RAD51C* mutation (c.577C>T). **d**, Diagram of the predicted *RAD51C* protein sequence changes caused by the primary (c.577C>T) and the secondary mutations (c.577_579delinsTGG, c.577C>A, c.574_577delinsGGCG, c.577_578delinsTT) detected in the progressing groin lymph node biopsy from the patient with the germline *RAD51C* mutation (c.577C>T). Examination of the parental OVCAR8, OVCAR8 *RAD51C* KO clone 2-130 and OVCAR8 *RAD51C* KO clone 2-130 transduced with wild-type (WT), primary or secondary mutant *RAD51C* transcripts using: **e**, Cell viability assay after treatment with rucaparib for 6 days and **f**, γ H2AX and RAD51 foci formation 48 h post rucaparib (10 μ M) exposure: γ H2AX foci are observed at the sites of DNA damage, and RAD51 foci are observed at the sites of HR pathway repair. **g**, Quantification of RAD51 foci formation in geminin positive cells (Mean \pm SEM). OVCAR8 *RAD51C* KO cells were transfected with plasmids expressing the WT, primary or secondary mutant *RAD51C* transcripts. The response of these cells to 10 μ M rucaparib was compared after 48 hr to parental OVCAR8 cell line or OVCAR8 *RAD51C* KO clone. n=8 (4 fields of view from 2 independent experiments) for each cell type and treatment. ***, p<0.001. **h**, *RAD51C* secondary mutants restore HR as well as WT *RAD51C* in *RAD51C*-mutant cells. *RAD51C*^{-/-} MCF10A cells containing the DR-GFP reporter were infected with an I-SceI expressing lentivirus and cultured for 48 hours. GFP+ cells were quantified by flow cytometry. n \geq 4 independent experiments. ***, p<0.001. **i**, *RAD51C* secondary mutants

restore RAD51C-R193* yeast-2-hybrid (Y2H) interactions with RAD51C binding partners RAD51B and XRCC3. *RAD51C* and the corresponding mutants were cloned into the yeast-2-hybrid (Y2H) plasmids expressing the *GAL4* activating domain (AD) whereas RAD51B and XRCC3 were cloned into *GAL4* binding domain (BD) expressing plasmids. Empty AD and BD vectors were used as negative controls. A Y2H interaction was observed as growth on medium lacking histidine, leucine, and tryptophan (Interaction), whereas equal cell loading was observed on medium lacking leucine and tryptophan (Control). PD, progressive disease; PR, partial response.

Figure 2. Tumor heterogeneity analysis of the post-progression biopsy with secondary *RAD51C* mutations. **a**, Model of the post-progression groin lymph node biopsy cores collected for analysis. Two post-progression core biopsies of the enlarging lymph node were obtained. Core 1 was used for genomic DNA analysis. Two ends of core 2, annotated as sections 1 and 2, were used for separate DNA extractions and subsequent analysis. The middle section of the core 2 was used to generate PDX, where 6 small pieces were subcutaneously transplanted into the recipient mice. The left-over section of core 2 adjacent to the section 1 was frozen in OCT and then sectioned for direct PCR library preparation. **b**, Variant allele frequencies detected by sequencing in the pre-treatment and post-progression biopsies, and in the generated PDX samples. Deep amplicon sequencing of *RAD51C* exon 4 (minimum coverage of 10,000x) was performed on these cores and on three recipient first passage (T1) mice. Whilst the first core analyzed contained all four *RAD51C* secondary mutations, only two of these were detected in the second core, which was used to generate the PDX. Spatial heterogeneity was even observed within the second core biopsy, with the c.577C>A mutation evident on one side of the core biopsy and at decreasing frequency towards the center of the core. The other side of the core biopsy predominately contained the c.574_577delinsGGCG mutation, as did PDX tumors expanded in three T1 mice implanted with tissue from the same core. PDX T2 was treated with 450mg/kg rucaparib for 2 weeks. **c**, Circos plot of the copy number alterations detected by WGS in the post-progression biopsy (Core 2 Section 2)

showing high levels of genomic instability. Losses are depicted in red, and gains in blue. **d**, Circos plot of copy number alterations detected by WGS in the two analyzed PDX tumor samples obtained from the post-progression biopsy (Core 2 Section 2). The outer and inner copy number tracks show the two analyzed tumor samples, while the middle track shows differences between them. **e**, *RAD51C* FISH assay of the post-progression biopsy (Core 2 OCT block for serial sectioning) and the PDX sample treated with 450mg/kg for 2 weeks. Arrows point to the cells with three distinct signals visible for the post-progression biopsy and the PDX sample.

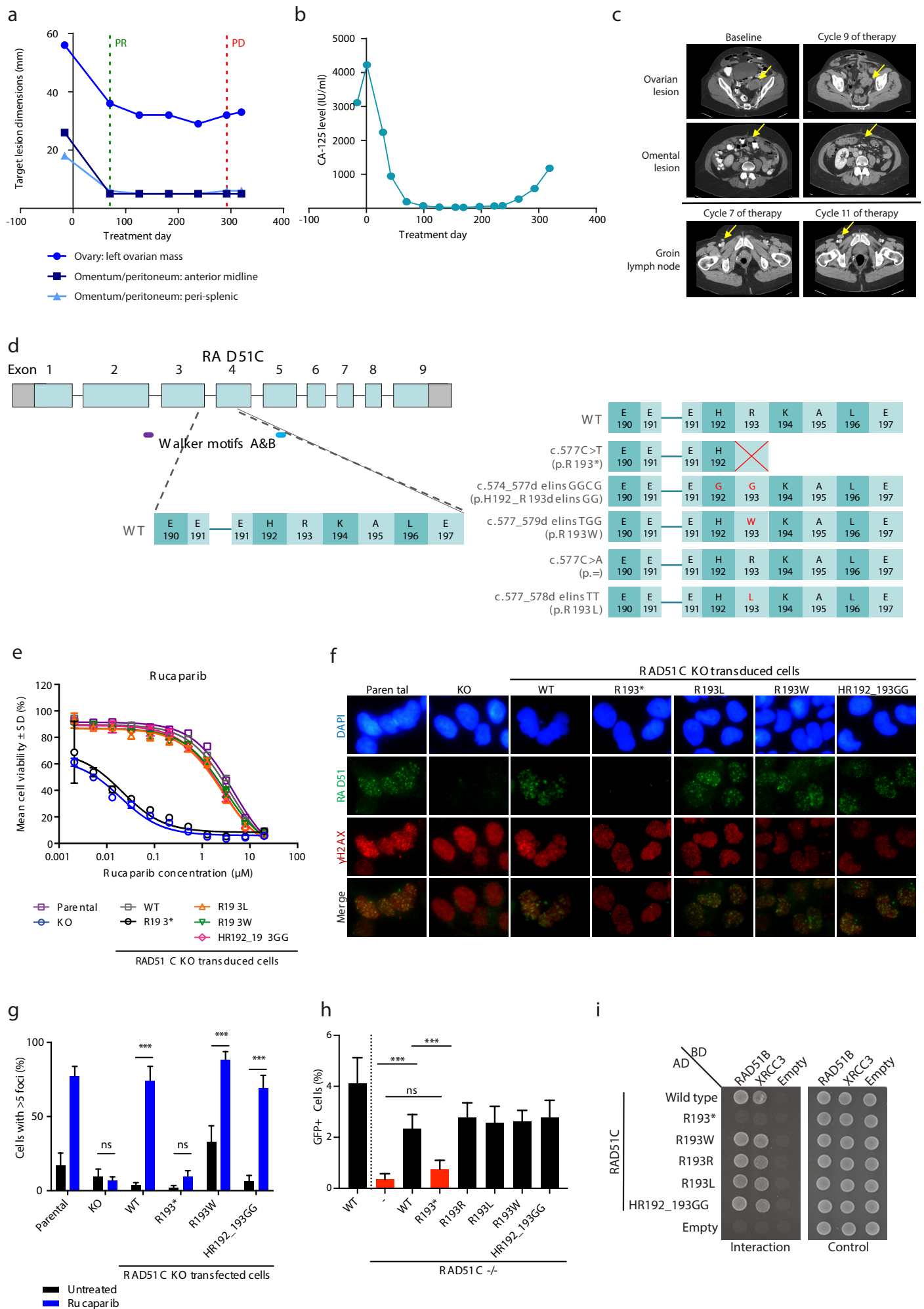
Figure 3. Identification and functional assessment of *RAD51D* secondary mutation identified in the post-progression biopsy in case 6. **a**, RECIST measurements of three tumor deposits in the patient with a germline *RAD51D* mutation (c.770_776del). The metastasis in the left lobe of the liver was biopsied prior to treatment. Surgery was performed following progression on rucaparib, in order to remove the enlarging splenic lesion. The tumor deposit in the liver, which was still responding to rucaparib treatment, was also excised. **b**, Serum CA-125 levels monitored during the ARIEL2 Part 1 trial in the patient with the germline *RAD51D* mutation (c.770_776del). **c**, Computed tomography (CT) images obtained prior to, during and following treatment of the patient with the germline *RAD51D* mutation (c.770_776del). **d**, Diagram of the predicted *RAD51D* protein sequence changes caused by the primary (c.770_776del) and the secondary mutation (c.770_776delinsA) detected in the patient with the germline *RAD51D* mutation. **e**, *In vitro* response to rucaparib in parental CHO cell line, CHO *RAD51D* KO clone and CHO *RAD51D* KO clone transduced with WT, primary or secondary mutant *RAD51D* transcripts after treatment for 6 days. **f**, *In vitro* response to rucaparib in parental PEO4 cell line, PEO4 cells with the homozygous frameshift *RAD51D* mutation (c.762_763del) and PEO4 cells with the homozygous secondary *RAD51D* mutation (c.770_776delinsA) after treatment for 7 days. **g**, *RAD51* foci formation 48 h post rucaparib (10 μ M) exposure in geminin positive cells in parental PEO4, PEO4 cells with the

homozygous frameshift *RAD51D* mutation (c.762_763del) and PEO4 cells with the homozygous secondary *RAD51D* mutation (c.770_776delinsA). SD, stable disease.

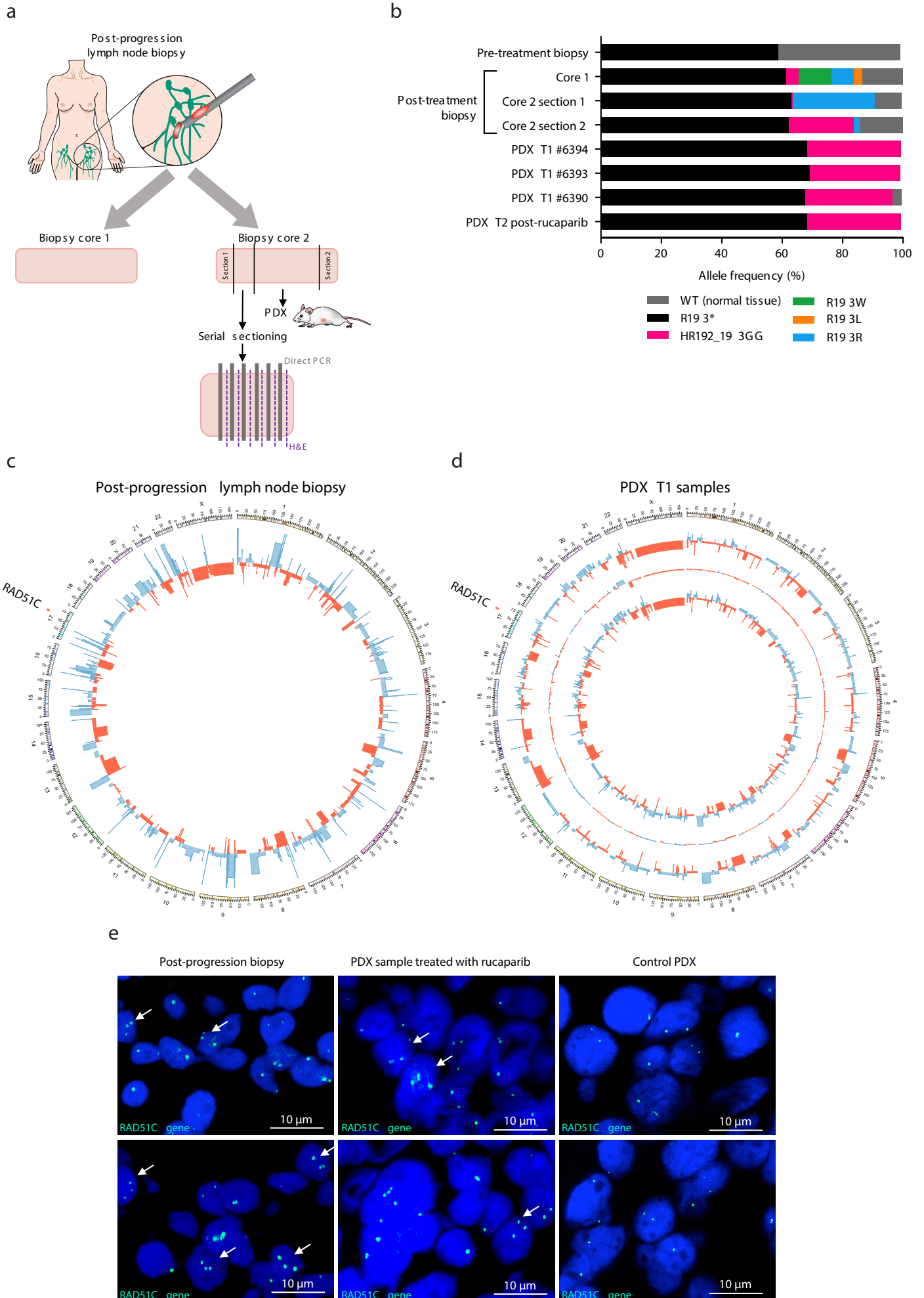
Table 1

Subject ID	# of prior treatment regimens	RECIST, CA-125 response	PFS (mo)	Archival tumour sample					Pre-treatment tumour sample					Post-progression tumour biopsy sample				
				Estimated tumour purity#	HR gene mutations detected HGVS nomenclature	Coverage	Mutation frequency	LOH	Estimated tumour purity#	HR gene mutations detected HGVS nomenclature	Coverage	Mutation frequency	LOH	Estimated tumour purity#	HR gene mutations detected HGVS nomenclature	Coverage	Mutation frequency	LOH
1	2	Partial response, CA-125 response	25.2	34%	<i>BRCA1</i> : c.1175_1214del, p.(L392Q6*5) GL	897	67%	yes	60%	<i>BRCA1</i> : c.1175_1214del, p.(L392Q6*5) GL	855	84%	yes	56%	<i>BRCA1</i> : c.1175_1214del, p.(L392Q6*5) GL <i>BRCA1</i> : c.1129_1251del, p.(S377_N417del) 2° MUT	991 1388	68% 31%	no no
2	1	Stable disease, CA-125 NE	22	14%	<i>BRCA1</i> : c.5346G>A, p.(W1782*) GL	524	57%	yes	73%	<i>BRCA1</i> : c.5346G>A, p.(W1782*) GL	542	77%	yes	56%	<i>BRCA1</i> : c.5346G>A, p.(W1782*) GL	534	70%	yes
3	1	Partial response, CA-125 response	16.4	91%	<i>BRCA1</i> : c.1892_1893del, p.(L631Q6*4) SOM	246	80%	yes	50%	<i>BRCA1</i> : c.1892_1893del, p.(L631Q6*4) SOM	796	33%	yes	20%	<i>BRCA1</i> : c.1892_1893del, p.(L631Q6*4) SOM <i>BRCA1</i> : c.1279_2139del, p.(E427_S713del) 2° MUT	1108 1215	10% 7%	no no
4	2	Partial response, CA-125 NE	12.8	62%	<i>BRCA1</i> : c.2043dup, p.(N682*) GL	768	73%	yes	<i>Tissue not suitable for analysis</i>					58%	<i>BRCA1</i> : c.2043dup, p.(N682*) GL <i>BRCA1</i> : c.1835_1964del; 2043dup, p.(R612_S681delins27) 2° MUT <i>BRCA1</i> : c.4096+1G>A 2° MUT	870 823 742	60% 15% 9%	no no no
5	1	Partial response, CA-125 response	9.6	80%	<i>RAD51C</i> : c.577C>T, p.(R193*) GL	656	92%	yes	36%	<i>RAD51C</i> : c.577C>T, p.(R193*) GL	711	73%	yes	66%	<i>RAD51C</i> : c.577C>T, p.(R193*) GL <i>RAD51C</i> : c.577_579delinsTGG, p.(R193W) 2° MUT <i>RAD51C</i> : c.577C>A, p.(=) 2° MUT <i>RAD51C</i> : c.574_577delinsGGCG, p.(H192_R193delinsCG) 2° MUT <i>RAD51C</i> : c.577_578delinsTT, p.(R193L) 2° MUT	1050 1050 1050 1021 1050	63% [^] 10% [^] 7% [^] 3% [^] 2% [^]	no no no no no
6	2	Stable disease, CA-125 response	11	91%	<i>RAD51D</i> : c.770_776del, p.(G258S6*50) GL	247	89%	yes	<i>Tissue not suitable for analysis</i>					91%	<i>RAD51D</i> : c.770_776delinsA, p.(S257_R259delinsK) 2° MUT†	418	90%	yes
7	1	Partial response, CA-125 response	16.7	85%	<i>CDK12</i> : c.264delC, p.(F89S6*3)	901	83%	yes	50%	<i>CDK12</i> : c.264delC, p.(F89S6*3)	712	50%	yes	74%	<i>CDK12</i> : c.264delC, p.(F89S6*3)	1027	75%	yes
8	1	Progressive disease, CA-125 NE	3.7	30%	<i>Nil found</i>				<i>Tissue not suitable for analysis</i>					39%	<i>Nil found</i>			
9	3	Stable disease, CA-125 NE	5	50%	<i>Nil found</i>				60%	<i>Nil found</i>				43%	<i>Nil found</i>			
10	2	Stable disease, CA-125 no response	3.5	73%	<i>Nil found</i>				<i>Tissue not suitable for analysis</i>					52%	<i>Nil found</i>			
11	2	Stable disease, CA-125 NE	18.2	90%	<i>Nil found</i>				78%	<i>Nil found</i>				66%	<i>Nil found</i>			
12	1	Stable disease, CA-125 no response	14.5	20%	<i>Nil found</i>				10%	<i>Nil found</i>				20%	<i>Nil found</i>			

Kondrashova et al Figure 1



Kondrashova et al Figure 2



Kondrashova et al Figure 3

